ELSEVIER

Enzyme and Microbial Technology 30 (2002) 752–757

ENZYME and MICROBIAL TECHNOLOGY

www.elsevier.com/locate/enzmictec

Effect of metal ions on the production of isomeric 9,10,13 (9,12,13)-trihydroxy-11*E* (10*E*)-octadecenoic acid from linoleic acid by *Pseudomonas aeruginosa* PR3

Hakryul Kim[a,*], Yong-Suk Jang[b], Ching T. Hou[a]

[a]*Microbial Genomic and Bioprocessing Research Unit, National Center for Agricultural Utilization Research, ARS, USDA, Peoria, IL, USA*
[b]*Division of Biological Sciences and The Institute for Molecular Biology and Genetics, Chonbuk National University, Chonju 561–756, Korea*

Received 20 July 2001; received in revised form 12 September 2001; accepted 2 October 2001

Abstract

Hydroxy fatty acids have gained important attentions because of their special properties such as higher viscosity and reactivity compared with other non-hydroxy fatty acids. Previously we reported that a novel bacterial strain *Pseudomonas aeruginosa* PR3 converted linoleic acid to the equimolar mixture of two compounds, 9,10,13- trihydroxy-11(*E*)-octadecenoic acid (9,10,13-THOD) and 9,12,13-trihydroxy-10(*E*)-octadecenoic acid (9,12,13-THOD) which showed anti-fungal activities (Kim, H. Gardner, H.W., Hou, C.T., *J. Ind. Microbiol. Biotechnol.* 2000, **25,** 109–115). In this study we report for the first time the effect of several metal ions as catalytic agents of lipid peroxidation on the production of total THODs by PR3. Among eight different metal ions tested, Fe^{+2} and Cu^{+2} were effective to produce THODs. However bacterial growth was not significantly affected by the existence of metal ions tested. Fe^{+2} ion requirement was specific to THOD production from linoleic acid but not to other incorporation of dihydroxyl group on oleic acid and ricinoleic acid by PR3.

Keywords: Bioconversion; Hydroxy fatty acid; Metal ion effect; *Pseudomonas*

1. Introduction

Hydroxy fatty acids are important industrial materials because the hydroxyl group on fatty acid gives fatty acid special properties such as higher viscosity and reactivity compared with other non-hydroxylated fatty acids [1]. The hydroxy fatty acids are used in a wide range of industrial products including resins, waxes, nylons, plastics, lubricants, cosmetics, and additives in coatings and paintings [2]. Of the hydroxy fatty acids reported, 9,10,11-trihydroxy-12-octadecenoic acid (9,10,11-THOD), 9,10,13-trihydroxy-11(*E*)-octadecenoic acid (9,10,13-THOD), and 9,12,13-trihydroxy-10(*E*)-octadecenoic acid (9,12,13-THOD) have gained special attention because they were isolated from plants with strong anti-fungal activity [3–6]. There are some other reports about the production of trihydroxy fatty acids from various sources with biologic significances [7–12]. However, although the biologic significances of these THODs were reported, their production in nature were rare and restricted mostly in plant system, and the compounds reported so far were all produced in trace amounts. During the past decades lots of effort were focused upon the microbial production of the hydroxy fatty acids using various fatty acid substrates.

The metabolic pathways involved in the conversion of linoleic acid to trihydroxy fatty acids were well studied. Enzymatic conversion of lipid hydroperoxides(LOOH), products of reactions catalyzed by lipoxygenase, have been reported in many higher plants [3–5,13–14]. Conversion of linoleic acid hydroperoxide by soybean lipoxygenase generated trihydroxy-, hydroperoxydihydroxy-, hydroxyepoxy-octadecenoate [11]. Although there detected many intermediates in the conversion of linoleic acid to THODs, overall pathway for THOD production from linoleic acids are believed to be via the generation of linoleic acid hydroperoxide by lipoxygenase and further decomposition into THODs. Decomposition of LOOH requires the presence of bivalent metal ions, e.g. iron or copper since these ions are known to be required to generate intermediate radicals ($LO^{\cdot}$) leading to the formation of hydroxyl group [15]. Iron ions

* Corresponding author. Fax: +82-63-270-4312.
E-mail address: hakrkim@moak.chonbuk.ac.kr (H. Kim).

0141-0229/02/$ – see front matter
PII: S0141-0229(02)00053-4

are also known for decades to be involved as an essential catalytic factor in lipoxygenases for their catalytic activity [16,17].

Previously, we reported that a novel bacterial strain *Pseudomonas aeruginosa* PR3 was able to convert unsaturated fatty acids to dihydroxy fatty acids [18–21] and linoleic acid to the equimolar mixture of 9,12,13-THOD and 9,10,13-THOD [22]. In this paper we report for the first time the effect of several metal ions as catalytic agents on the production of total THODs by PR3.

2. Materials and methods

2.1. Microorganisms

Pseudomonas aeruginosa NRRL strain B-18602 (PR3) isolated from water sample of a hog farm near Peoria, IL, USA was grown at 28°C aerobically in a 125 ml Erlenmeyer flask containing 50 ml of standard medium with shaking at 200 rpm. The standard medium used hereafter contained per liter 4 g dextrose, 2 g K_2HPO_4, 2 g $(NH_4)_2HPO_4$, 1 *g* NH_4NO_3, 0.5 g yeast extract, 0.014 g $ZnSO_4$, 0.01 g $FeSO_4.7H_2O$, and 0.01 g $MnSO_4 \cdot 7H_2O$. For the study of metal ion effect on THOD production, all the defined metal ion salts of standard medium were replaced with single metal ion-sulfate salt as specified elsewhere. The concentration of metal ion mentioned elsewhere in the text indicates the concentration of the metal ion newly added in the basal medium. Basal medium represents the medium that contains yeast extract but no defined metal ions. The medium was adjusted to pH 7.0 with diluted phosphoric acid. Standard condition referred in the text represented those mentioned above. Cultures were maintained on agar slant with the medium mentioned above except for the addition of 1.5% agar.

2.2. Chemicals

Linoleic acid and methyl ester of elaidic acid with 99^+ % purity by gas chromatography were purchased from NU-Check-Prep Inc. (Elysian, MN). Mixture of trimethylsilylimidazole (TMSI) and pyridine (1:4 v/v) was purchased from Supelco Inc. (Bellefonte, PA). All other chemicals were reagent grade and were used without further purification. Other chemicals were purchased from Sigma, unless mentioned otherwise.

2.3. Bioconversion

Linoleic acid (0.5 g) as substrate was added to a 24 h-old culture in the standard or modified medium followed by continued incubation for an additional 72 h. At the end of the cultivation, the culture broth was acidified to pH 2 with 6 N HCl followed by immediate extraction twice with an equal volume of ethyl acetate and diethyl ether. The solvent was evaporated from the combined extracts with a rotary evaporator.

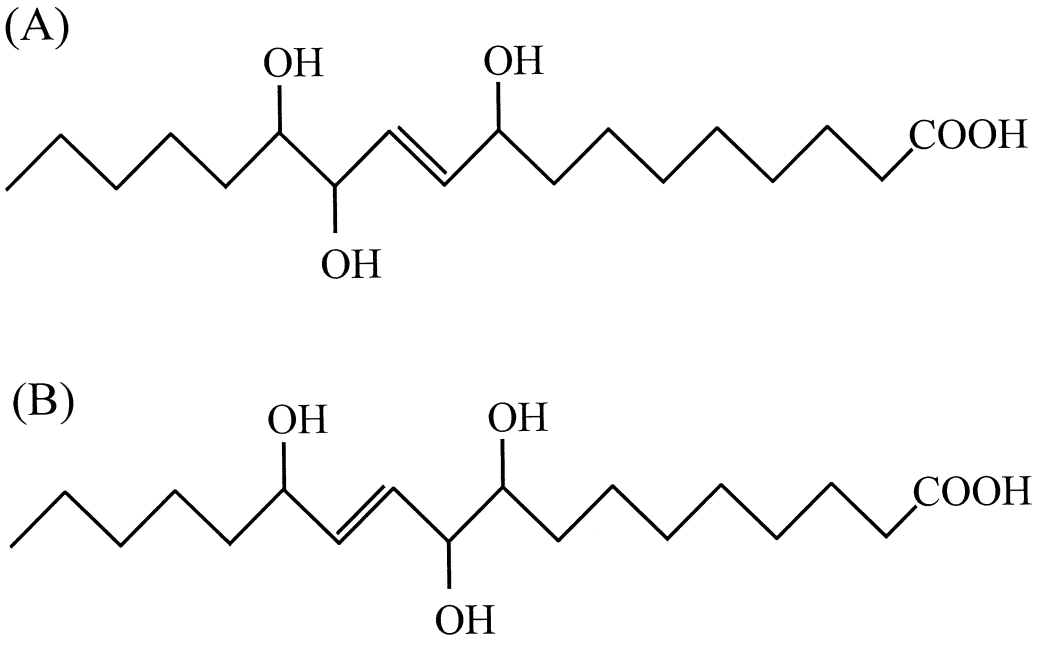
2.4. Analysis of products

Products in crude extract were analyzed and identified by GC/mass spectrometry (GC/MS). The crude samples were first methylated with diazomethane for 1 min at room temperature. After the solvent was evaporated under the nitrogen gas flow, the methyl esters were derivatized with the mixture of TMSI and pyridine (1:4 v/v) for at least 20 min at room temperature. The TMSI-derivatized sample was analyzed with a Hewlett Packard (Avondale, PA) 5890 GC coupled to a Hewlett Packard 5972 Series Mass Selective Detector. The column outlet was connected directly to the ion source. Separations were carried out in a methylsilicone capillary column with 30 m x 0.25 mm I.D., 0.25 μm film thickness (Supelco Inc., Bellefonte, PA). GC was run with a temperature gradient of 20°C/min from 70°C to 200°C, holding 1 min at 200°C, and then 0.7°C/min to 240°C, followed by holding for 15 min at 240°C (helium flow rate = 0.67 ml/min). For quantitative analysis, methyl ester of elaidic acid was added to the sample as an internal standard prior to TMSI derivatization. Internal standard method was confirmed by gravimetric measurement of product. Chemical structure of the analyzed product was determined with the electron-impact mass spectra obtained with GC/MS. The values presented in each experiment of this study are averages of the duplicate. Error range was within 10% of average value.

3. Results and discussion

3.1. Effect of metal ions on THOD production from linoleic acid by PR3

It has been reported that lipid peroxide molecules(LOOH) were decomposed to radicals in the presence of bivalent metal ions, in particular, iron or copper since these ions were known to be required to generate intermediate radicals ($LO^{\cdot}$) leading to the formation of hydroxyl group on fatty acid [15]. In our previous report, we demonstrated that a novel bacterial strain *Pseudomonas aeruginosa* PR3 could convert linoleic acid into the equimolar mixture of 9,12,13-THOD and 9,10,13-THOD with about 45% yield [22]. The structure of 9,12,13- and 9,10,13-THODs are shown in Scheme 1. To address the effect of metal ions on the production of THODs by PR3, we prepared medium in which all the defined metal ions in the standard medium were replaced with 70 μM of single metal ion-sulfate salt (see Materials and Methods) and tested each medium to compare THOD production under the standard condition. As shown in Table 1, Fe^{+2} and Cu^{+2} ions among the eight metal ions tested were sufficiently effective to produce THOD compared to the control medium. In the case of copper ion, at a



Scheme 1. Structure of 9,12,13-THOD (A) and 9,10,13-THOD (B) produced from linoleic acid by PR3.

given concentration, THOD production was about 20% higher than that with control medium. However single use of iron ion at a given concentration was less effective by 30% for THOD production compared to the control. Except for Mn^{+2} ion which showed 6% relative productivity of THODs, other ions tested produced no THODs.

Lipoxygenase from soybean has been reported to convert linoleic acid to linoleic hydroperoxide which was important intermediate leading to the formation of THODs [11]. Soybean 15-lipoxygenase contains non-heme Fe(III) essential for its catalytic activity [16–17,23]. The Fe(III) center of lipoxygenases has been suggested to play a central catalytic role for activity, probably through the generation of a fatty acid radical which subsequently reacts with molecular oxygen to produce the hydroperoxide [24]. In addition, certain inhibitors of lipoxygenase reduced the active ferric enzyme to the inactive ferrous form [25,26]. However, lipid peroxidation of linoleic acid was induced by catalytic amounts either of Fe^{+2} or Fe^{+3} ions [27–29]. Since these two ions are easily convertible each other by oxidation and reduction, there could be always an equilibrium between them. In other report, oxidation of (13*S*, 9*Z*, 11*E*) 13-hydroxyperoxy-9,11-octadecadienoic acid (13S-HPODE), which is the products of linoleic acid peroxidation, proceeded much faster with air in the presence of equimolar amounts of Fe^{+2} ions than the case with Fe^{+3} ions [30]. Gardner et al. observed that trihydroxy fatty acids were generated by the action of catalytic amounts of Fe^{+2} on linoleic acid hydroperoxides [28]. These observations prompted us to assume that Fe^{+3} ions are required for lipoxygenase activity but Fe^{+2} ions are more favorable for oxidation of LOOH, hence suggesting that surplus amount of Fe^{+2} ions in the culture medium could sufficiently enable the production of THODs from linoleic acid by PR3. Therefore we used Fe^{+2} ions instead of Fe^{+3} ions in this study.

Table 1
Effect of metal ions on THOD production by PR3

Metal ions (70 μM)*	Total THOD‡ (mg/50 ml culture)	Cell density (O.D. @ 540 nm)
Control†	18.6	1.29
Fe^{+2}	13.0	1.54
Cu^{+2}	21.9	1.58
Zn^{+2}	nd	1.40
Mn^{+2}	1.1	1.30
Mg^{+2}	nd	1.48
Ca^{+2}	nd	1.67
Na^{+1}	nd	1.67
K^{+1}	nd	1.69

* All metal ions tested were supplied as sulfate salt.
† Standard medium was used as control.
‡ nd indicates not detectable.

3.2. Effect of Fe^{+2} ion concentration on the production of THODS by PR3

Since Fe^{+2} ion was shown to be required for THOD production from linoleic acid by PR3, we investigated the effect of varied Fe^{+2} ion concentrations on the production of total THODs from linoleic acid by PR3. THOD production showed non-linear increment in relation to the increase of Fe^{+2} ion concentration (Fig. 1). THOD production increased steadily resembling lag phase of cell growth curve up to 0.07 mM of Fe^{+2} ion concentration after which the production entered exponential increase up to 0.14 mM of

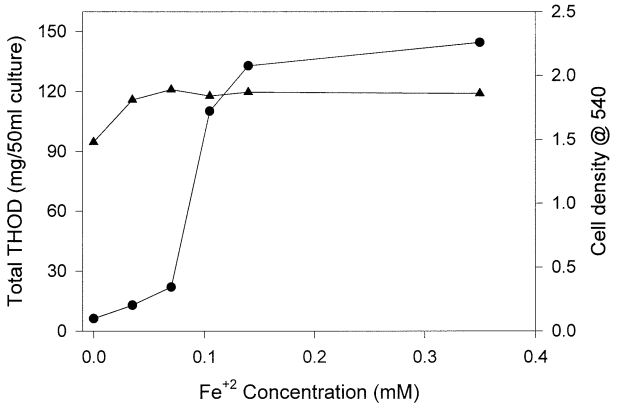


Fig. 1. Effect of Fe^{+2} ion concentrations on the production of total THODs from linoleic acid by PR3. All the defined metal ions in the standard medium were replaced with single ferrous sulfate. THOD was produced with 500 μg of linoleic acid as substrate for 72 h under standard condition and total THODs (circle) produced and extracted were quantified with methyl ester of elaidic acid being added to the sample as an internal standard prior to TMSI derivatization of extracts. Cell density (triangle) was expressed as the optical density measured at 540 nm.

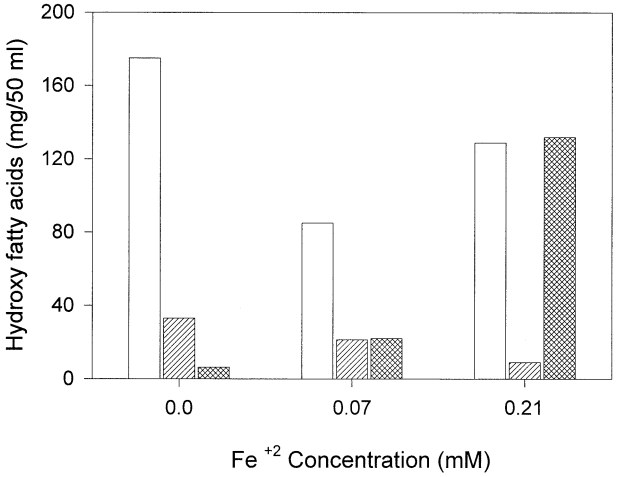


Fig. 2. Effect of Fe^{+2} ion concentrations on the production of hydroxy fatty acids from their corresponding substrates by PR3. All the defined metal ions in the standard medium were replaced with single ferrous sulfate at different concentrations. Other experimental conditions followed the one used in Fig. 1. Open bar represented DOD production from oleate, striated bar and meshed bar represented TOD production from ricinoleic acid and total THOD production from linoleic acid, respectively.

Fe^{+2} ions followed by saturation of THOD production. However, microbial growth was not significantly influenced by the concentration of Fe^{+2} ions tested. Even in the absence of defined metal ions at all, cell density marked up to 80% of the maximal density. From these results we came to a conclusion that the threshold concentration level of Fe^{+2} ions for the production of THODs from linoleic acid by PR3 existed between 0.07 mM and 0.14 mM. This concentration range accounted for the substrate/Fe^{+2} ion molar ratio of from 5100 to 2550. It is highly plausible to assume that existence of the threshold concentration level of Fe^{+2} ion for THOD production could possibly be raised by the preference of two iron ions Fe^{+2}/Fe^{+3} in the equilibrium for discrete reactions by the enzyme system involved in this event.

3.3. Effect of Fe^{+2} ion concentration on the production of hydroxy fatty acids from various substrates by PR3

PR3 used in this study has been reported to convert oleic acid and ricinoleic acid into 7,10-dihydroxy-8(E)-octadecenoic acid (DOD) and 7,10,12-trihydroxy-8(E)-octadecenoic acid (TOD), respectively [20,31]. In our previous reports, we postulated that oleic acid and/or ricinoleic acid-specific lipoxygenase rather than hydratase would be involved in the production of DOD and TOD from their corresponding substrate by PR3 [18,21]. Hence, we investigated the effect of Fe^{+2} ions on the production of DOD and TOD from their corresponding substrates in comparison with THOD production from linoleic acid by PR3 (Fig. 2). When Fe^{+2} ions were added at two different concentrations of 0.07 mM and 0.21 mM, THOD production increased greatly as expected. However, TOD production from ricinoleic acid linearly decreased with relatively low production level and DOD production from oleic acid fluctuated with its production in the absence of Fe^{+2} ion being even higher than that in the presence of iron.

These results demonstrated that the enzyme(s) involved in the production of THOD from linoleic acid would be different from the enzyme system involved in the formation of DOD and TOD from their corresponding substrates by PR3 and that the enzyme could be lipoxygenase. This was supported by the report that the key element of the substrate for the non-heme iron containing lipoxygenase was a 1,4-*cis*,*cis*-diene unit present in the structure of linoleic acid [23]. This structure is different from the single *cis* double bond found on C9,10 of oleate and ricinoleic acid. However, further work should be performed to identify the lipoxygenase in PR3.

3.4. Effect of Cu^{+2} ions on the production of total THOD by PR3

Copper ion was known to be one of the bivalent metal ions effective to decompose LOOH to radicals [15] and also concomitantly our primary result in this study demonstrated that the presence of Cu^{+2} ions was more effective for THOD production than the control medium (Table 1). Therefore, we investigated the effect of varied Cu^{+2} ion concentrations on the production of total THODs from linoleic acid by PR3. As shown in the Fig. 3, THOD production increased in proportion to the concentration of Cu^{+2} ion and the inclining pattern of THOD production over the increasing concentration of Cu^{+2} ions was similar to that with Fe^{+2} ions. However, the threshold level of Cu^{+2} ion concentration for THOD production appeared between 0.01 mM and 0.04 mM, and also maximum THOD production

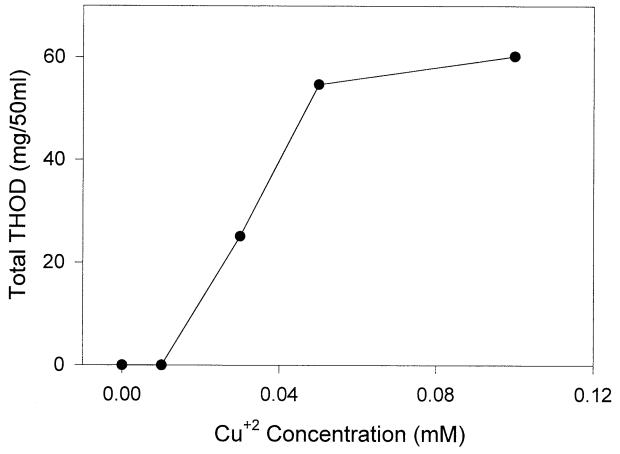


Fig. 3. Effect of Cu^{+2} ion concentrations on the production of total THODs from linoleic acid by PR3. All the defined metal ions in the standard medium were replaced with single cupric sulfate at different concentrations. Other experimental conditions followed the one used in Fig. 1.

level was lower than that with Fe^{+2} ions. These results suggested that Fe^{+2} ions were more efficient than Cu^{+2} ions for the production of THODs from linoleic acid by PR3 above the threshold level.

In summary, we studied for the first time the effect of metal ions on the production of trihydroxy fatty acids from linoleic acid by a bacterial strain *Pseudomonas aeruginosa* PR3. Fe^{+2} or Cu^{+2} ions were required for THOD production from linoleic acid by PR3. However, Fe^{+2} ions were more efficient than Cu^{+2} ions for the production of THODs and there existed threshold level of Fe^{+2} and Cu^{+2} ion concentration for THOD production by PR3 with the former being higher than the latter. The existence of threshold level of metal ion concentration suggested that THOD production from linoleic acid by PR3 in mediation with Fe^{+2} or Cu^{+2} ions was carried out probably by lipoxygenase through independent two or more catalytic steps.

Acknowledgment

We gratefully acknowledge the excellent technical assistance of AL Ashbury. She was student assistant from Bradley University in Peoria, IL, USA. This research was supported by the International Cooperation Research program of the Ministry of Science and Technology.

References

[1] Bagby MO, Calson KD. Chemical and biological conversion of soybean oil for industrial products. In: Fats for the future, Chichester: Ellis Horwood Limited Press, 1989. p. 301–7.
[2] Naughton FC. Production, chemistry, and commercial applications of various chemicals from castor oil. J Am Oil Chem Soc 1974;51:65–71.
[3] Kato T, Yamaguchi Y, Abe N, Uyehara T, Nakai T, Yamanaka S, Harada N. Unsaturated hydroxy fatty acids, the self-defensive substances in rice plant against rice blast disease. Chem Lett 1984;25: 409–12.
[4] Kato T, Yamaguchi Y, Abe N, Uyehara T, Namai T, Kodama M, Shiobara Y. Structure and synthesis of unsaturated trihydroxy C_{18} fatty acids in rice plant suffering from rice blast disease. Tetrahedron Lett 1985;26:2357–60.
[5] Kato T, Yamaguchi Y, Ohnuma SI, Uyehar T, Nmai T, Kodama M, Shibara Y. Structure and synthesis of 11,12,13-trihydroxy-9Z,15Z-octadecadienoic acids from rice plant suffering from rice blast disease. Chem Lett 1986;27:577–80.
[6] Masui H, Kondo T, Kojima M. An antifungal compound, 9,12,13-trihydroxy-(*E*)-10-octadecenoic acid, from *Colocasia antiquorum* inoculated with *Ceratocystis fimbriata.* Phytochem 1989;28:2613–5.
[7] Baur C, Grosch W, Wieser H. Jugel H. Enzymic oxydation of linoleic acid: formation of bittertasting fatty acids. Z Lebesm Unters-Forsxh 1977;164:171–6.
[8] Esterbauer H, Schauenstein E. Isomeric trihydroxy-octadecenoic acids formed upon enzymic oxidation of linoleic acid by barley flour. Monatshefte für Chemie 1977;108:963–72.
[9] Funk CD, Powell WS. Metabolism of linoleic acid by prostaglandin endoperoxide synthase from adult and fetal blood vessels. Biochim. Biophys Acta 1983;754:57–71.
[10] Galliard T, Phillips DR, Matthew JA. Enzymic reactions of fatty acid hydroperoxides in extracts of potato tuber. Biochim Biophys Acta 1975;409:157–71.
[11] Streckert G, Stan HJ. Conversion of linoleic acid hydroxperoxide by soybean lipoxygenase in the presence of guaiacol: identification of the reaction products. Lipids 1975;10:847–54.
[12] Ustunes L, Claeys M, Laekeman G, Herman AG, Vlietinck AJ, Ozer A. Isolation and identification of two isomeric trihydroxy octadecenoic acids with prostaglandin e-like activity from onion bulbs (Allium cepa). Prostaglandins 1985;29:847–65.
[13] Vick BA, Zimmerman DC. Oxidative systems for modification of fatty acids: the lipoxigenase pathway. In: The biochemistry of plants: a comprehensive treatise, vol. 9, Orlando, FL: Academic Press, 1987. p. 53–90.
[14] Zimmerman DC. A new product of linoleic acid oxidation by a flaxseed enzyme. Biochem Biophys Res Commun 1966;23:398–402.
[15] Gardner HW. Oxygen radical chemistry of polyunsaturated fatty acids. Free Radic Biol Med 1989;7:65–86.
[16] Schilstra MJ, Veldink GA, Vliegenthart JF. The dioxygenation rate in lipoxygenase catalysis is determined by the amount of iron (III) lipoxygenase in solution. Biochemistry 1994;33:3974–9.
[17] Zhang YY, Lind B, Radmark O, Samuelsson B. Iron content of human 5-lipoxygenase, effects of mutations regarding conserved histidine residues. J Biol Chem 1993;268:2535–41.
[18] Kim H, Gardner HW, Hou CT. 10(S)-hydroxy-8(*E*)-octadecenoic acid, an intermediate in the conversion of oleic acid to 7,10-dihydroxy-8(E)-octadecenoic Acid J Am Oil Chem Soc 1999;77:95–9.
[19] Hou CT, Bagby MO, Platner RD, Koritala SA. Novel compound, 7,10-dihydroxy-8(E)-octadecenoic acid from oleic acid by bioconversion. J Am Oil Chem Soc 1991;68:99–101.
[20] Hou CT, Bagby MO. Production of a new compound, 7,10-dihydroxy-8(E)-octadecenoic acid from oleic acid by *Pseudomonas* sp. PR3. J Ind Microbiol 1991;7:123–30.
[21] Kim H, Kuo TM, Hou CT. Production of 10,12-dihydroxy-8(E)-octadecenoic acid, an intermediate in the conversion of ricinoleic acid to 7,10,12-trihydroxy-8(*E*)-octadecenoic acid by *Pseudomonas aeruginosa* PR3. J Ind Microbiol Biotechnol 1999;24:167–72.
[22] Kim H, Gardner HW, Hou CT. Production of isomeric (9,10,13)-trihydroxy-11e(10E)-octadecenoic acid from linoleic acid by *Pseudomonas aeruginosa* PR3. J Ind Microbiol Biotechnol 2000;25:109–15.
[23] Veldink GA, Vliegenthart JF. Lipoxygenases, nonheme iron-containing enzymes. Adv Inorg Biochem 1984;6:139–61.

[24] Abeysinghe RD, Roberts PJ, Cooper CE, MacLean KH, Hider RC, Porter JB. The environment of the lipoxygenase iron binding site explored with novel hydroxypyridinone iron chelators. J Biol Chem 1996;271:7965–72.
[25] Clapp CH, Banerjee A, Rotenberg SA. Inhibition of soybean lipoxygenase 1 by N-alkylhydroxylamines. Biochemistry 1985;24:1826–30.
[26] Kemal C, Louis-Flamberg P, Krupinski-Olsen R, Shorter AL. Reductive inactivation of soybean lipoxygenase 1 by catechols: a possible mechanism for regulation of lipoxygenase activity. Biochemistry 1987;26:7064–72.
[27] Gardner HW, Weisleder D, Kleiman R. Formation of *trans*-12,13-epoxy-9-hydroperoxy-*trans*-10-octadecenoic acid from 13-L-hydroperoxy-*cis*-9, *trans*-11-octadecenoic acid catalyzed by either a soybean extract or cystein-$FeCl_3$. Lipids 1976;13:246–52.
[28] Gardner HW, Kleiderman R, Weisleder D. Homolytic decomposition of linoleic acid hydroperoxide:identification of fatty acid products. Lipids 1974;9:696–706.
[29] Dix TA, Marnett LJ. Conversion of linoleic acid hydroperoxide to hydroxy, keto, epoxyhydroxy, and trihydroxy fatty acids by Hematin. J Biol Chem 1985;260:5351–7.
[30] Spiteller P, Spiteller G. Strong dependence of the lipid peroxidation product spectrum whether Fe^{2+}/O_2 or Fe^{3+}/O_2 is used as oxidant. Biochim Biophys Acta 1998;1392:23–40.
[31] Kuo TM, Manthey LK, Hou CT. Fatty acid bioconversion by *Pseudomonas aeruginosa* PR3. J Am Oil Chem Soc 1998;75:875–9.